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Effect of Polymyxin on the Bacteriophage Receptors of the Cell Walls of Gram-Negative Bacteria

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Treatment of gram-negative bacteria with lethal doses of polymyxin B and colistin resulted in the formation of projections of the outer layer of the cell wall. Phages T3, T4, and T7, which use wall lipopolysaccharide as receptors, were specifically prevented from adsorbing to *Escherichia coli* B cells treated with polymyxin, whereas phages T1, T2, T5, and T6 were not. In the systems of phage P22C-*Salmonella typhimurium* LT2 and phage C21-*S. typhimurium* variant SL1069, the phage were prevented from adsorbing to the host cell treated with the antibiotics. Electron microscopic observations show that phage T2 adsorbed irreversibly to the normal smooth surface between the projections on the outer layer caused by the drug treatment. These results indicate that lipopolysaccharide is affected by polymyxin functionally and morphologically, but lipoprotein is not. The purified lipopolysaccharide showed a ribbon-like structure when viewed face on and showed trilamellar structure when viewed edge on. The lipopolysaccharide from *E. coli* B was irreversibly adsorbed by phages T3, T4, and T7, but not phage T2. Often, phage T4 adsorbed to both sides of the lipopolysaccharide strand at comparable distances. Phage P22C adsorbed through the spikes of the tail-plates to the lipopolysaccharide from *S. typhimurium* LT2. Lipopolysaccharide which was treated with low doses of the drug (2.5 to 6.25 μg of polymyxin B per ml to 100 μg of lipopolysaccharide per ml) turned into the coiled form and was partially broken down into short segments with coiled form. The loosely coiled lipopolysaccharide retains both its function as the receptor and its trilamellar structure. Treatment with high doses of the drug (12.5 to 25 μg of polymyxin B per ml to 100 μg of lipopolysaccharide per ml) caused the collapse of the trilamellar structure of the strand. These collapsed lipopolysaccharides became flat and fused with each other, making an amorphous mass, and finally they were broken into small collapsed fragments.

Polymyxin coats the cell envelope, consisting of the cell wall and the cytoplasmic membrane, of gram-negative bacteria and destroys its normal function as a selective permeability barrier (5, 6, 16). Several morphological studies have been performed to elucidate the mechanism of polymyxin action (2, 3). We have previously shown that projections of the outer layer of the cell wall and discontinuity of the cytoplasmic membrane were produced by polymyxin B and colistin treatment at a time soon after exposure (11). It is well known that lipoprotein and lipopolysaccharide (LPS) are two major components of the outer layer of the cell wall of gram-negative bacteria. Recently, Lopes and Inniss (13) showed by electron microscopy that the LPS extracted from *Escherichia coli* was broken down into short sections when it was exposed to polymyxin. However, it was uncertain whether or not polymyxin affected any other surface component of the cell wall, e.g. lipoprotein. The cell wall has a mosaic of phage-specific receptors. For instance, the receptors for phages T3, T4, and T7 on *E. coli* B have been shown to be LPS, and the ones for phages T2 and T6 have been shown to be lipoprotein (26). To determine which class of these phage receptors is affected by polymyxin could offer a useful index to the mode of action of polymyxin. Our experiments were designed for this purpose.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The bacterial strains used in this work were *E. coli* B, *Salmonella*

typhimurium LT2 and variant SL1069 (rfb) furnished by T. Watanabe, Keio University, Japan. Bacteriophages used were T1, T2h, T3, T4B, T6, and T7 furnished by M. Sekiguchi, Kyushu University, Japan, phage T5 furnished by H. Ozeki, National Institute of Health of Japan, phage C21 kindly provided by B. A. D. Stocker, Stanford, University, U.S.A., and phage P22 clear mutant (P22C) provided by T. Watanabe.

Materials and media. Polymyxin B sulfate (PLB) was supplied by Taito Pfizer Co., Tokyo, Japan, and colistin sulfate (CL) by Kaken Co., Tokyo, Japan. The medium for culture of bacteria and propagation of bacteriophages was M-9 medium supplemented with 0.5% Difco Casamino Acids. For the culture of SL1069 and propagation of phage C21, Lennox broth was used (12). For the plaque counting of T-series and P22C phages, Difco nutrient agar was used, and Lennox agar was used for C21.

Extraction and purification of the cell wall LPS. Overnight cultures of each bacterium in 10 liters of aerated Difco nutrient broth were harvested by centrifugation and washed twice with distilled water. After destruction of the bacteria by a cell fractionator (Ribi), the purified cell wall fractions were obtained by differential centrifugation at 16,000 × *g* for 30 min. LPS from each cell wall fraction was extracted by the hot phenol-water method of Westphal et al. (28). The LPS extract was repeatedly precipitated and washed with ethanol and then lyophilized.

Measurement of efficiency of adsorption. To 0.9 ml of cell suspension containing about 5×10^8 viable cells per ml, 0.1 ml of a saline solution containing 100 μg of PLB or CL per ml was added, and the mixture was incubated at 37 C for 10 min. One sample received 0.1 ml of saline as control. Then 1 ml of each bacterial suspension was mixed with 1 ml of prewarmed phage suspension at appropriate multiplicity of infection (MOI). After incubation for 10 min, 0.1 ml of the mixture was diluted in 9.9 ml of saline containing 0.3 ml of chloroform, and the number of unadsorbed phage was estimated by a plaque counting method (1). The mixture of phage T5 and cells was incubated for 20 min to allow sufficient adsorption. The ratio of adsorbed phage to the number of phage in the original suspension was calculated as adsorption efficiency.

Electron microscopy. For electron microscopy of thin sections, bacterial cells which adsorbed phage for 10 min at MOI of about 100 to 200 were doubly fixed by 1% glutaraldehyde in phosphate buffer and Kellenberger and Ryter's OsO_4 fixative and then treated by 0.5% uranyl acetate (9). After dehydration through an alcohol series, the specimens were embedded in Epon 812 (14). Thin sections were made with a Sorvall Porter-Blum MT-2 ultramicrotome and then stained by lead citrate (24). Negative and positive staining was performed with 2% phosphotungstate or 2% uranyl acetate. The materials were examined with a JEM-7 and a JEM-100B electron microscope.

RESULTS

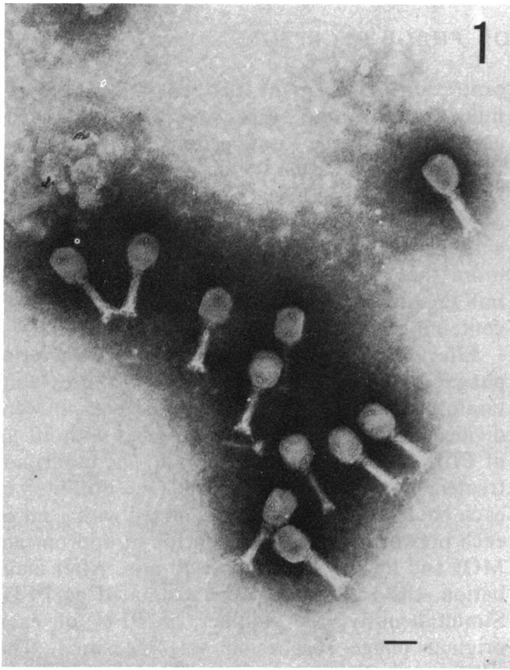
Effect of polymyxin on the activity of bacteriophage. To determine whether or not polymyxin affects the plaque-forming activity of phage, the phage suspension with PLB or CL at final concentration of 10 μg/ml was kept at 37 C for 10 min, and then the suspension was diluted and assayed for plaque-forming units (PFU). No effect of PLB or CL on the activity of phage could be detected. No morphological changes could be observed by negative staining in phage treated with PLB or CL. For instance, after treatment more than 95% of the phage T4 had extended tail sheaths and deoxyribonucleic acid (DNA)-containing heads (Fig. 1).

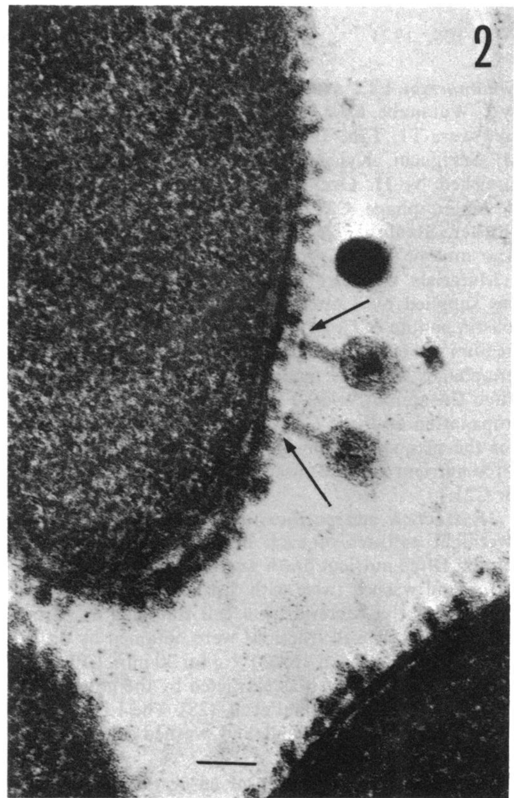
Effect of polymyxin on adsorption of bacteriophage to the host cell receptors. Cultures containing about 5×10^8 viable cells per ml were divided in half. One half was treated with 10 μg of PLB or CL per ml for 10 min. The other, not treated, was used as a control. One milliliter of each bacterial suspension was mixed with 1 ml of each prewarmed phage suspension at appropriate MOI and incubated for 10 or 20 min. After incubation, the free phage were estimated as PFU. Simultaneously, the number of PFU of each original phage suspension was measured. The adsorption efficiency was calculated as follows:

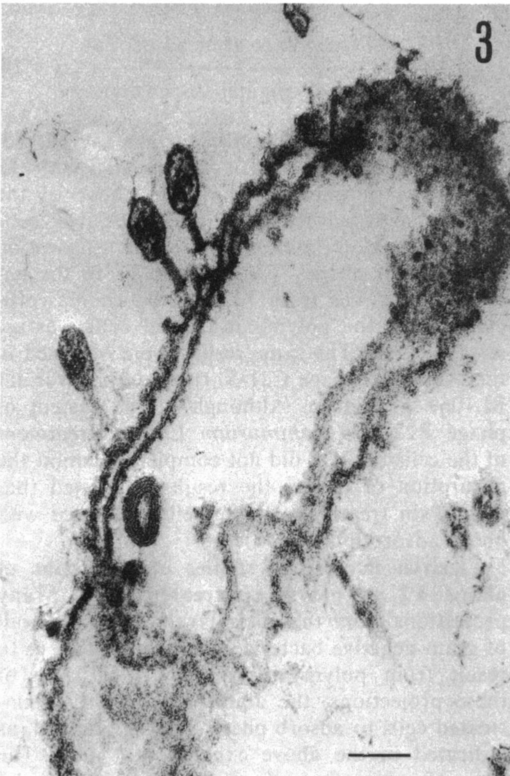
$$1 - \left(\frac{\text{PFU of free phage after adsorption}}{\frac{1}{2}\ \text{PFU of original suspension}}\right) \times 100$$

The rates of adsorption of phages T1, T2, T5, and T6 to the cells of *E. coli* B treated with the antibiotics were fairly similar to those of the same phages adsorbing to the untreated cells (Table 1). It is interesting that the adsorption efficiency of phages T3, T4, and T7 to the untreated cells was from 70 to 90%, whereas efficiency with the polymyxin-treated cells was essentially zero. The same results were obtained in the system of phage C21-*S. typhimurium* variant SL1069 (Table 1). Although in the system of phage P22C-*S. typhimurium* LT2 pretreatment of the cells by PLB did not completely inhibit the adsorption of phage, the results suggested that polymyxin treatment of the cells interfered with phage adsorption.

Electron microscope studies of adsorption of phage T2 to polymyxin-treated cells. Many projections from the outer layer of the cell wall of gram-negative bacteria were observed by us to result from polymyxin treatment. In spite of these projections, the ability of the polymyxin-treated cells to adsorb phage T2 was retained, as indicated in the above experimental data. The sites of adsorption of phage T2 to the polymyxin-treated cells were detected by electron microscopy. Many phage which adsorbed to the untreated cells had empty heads and contracted tail sheaths. Long tail fibers extended laterally from the baseplate, and tail core needles attached to the cell wall. When the phage were







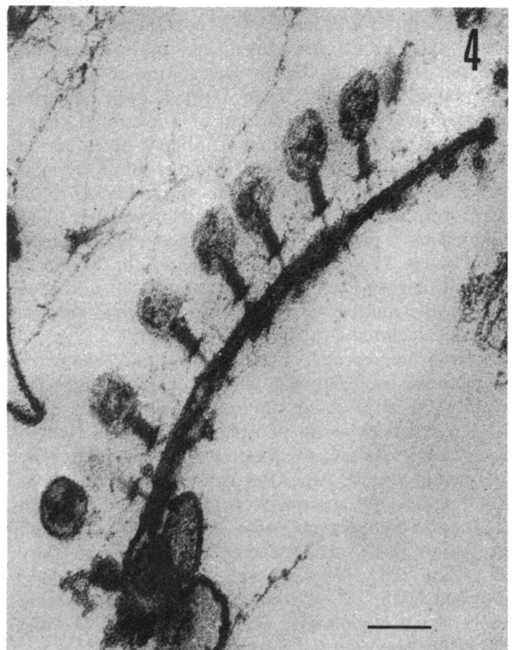


FIG. 1. *Negatively stained phage T4 treated with 10 μg of polymyxin B per ml for 10 min. Markers represent 100 nm.*

FIG. 2. *Thin section of polymyxin B-treated E. coli B infected by phage T2. On the normal cell surface between the projections, irreversibly adsorbed phage with empty heads and contracted sheaths are seen (arrows).*

FIG. 3. *Thin section of E. coli B with phage T2 adsorbed before polymyxin B treatment. No projections were seen at the sites where the phage attached irreversibly.*

FIG. 4. *Thin section of E. coli B with phage T4 adsorbed before polymyxin B treatment. Neither at the site of phage adsorption, nor between the sites of phage adsorption, could any structural alterations such as projections be seen.*

TABLE 1. *Effect of polymyxin on adsorption of phage to host cells*

Phage	Host cell	MOI	Adsorption efficiency[a] (%)			
			Polymyxin B		Colistin	
			None	10 μg/ml	None	10 μg/ml
T1	*E. coli* B	2.2	65.0	40.0	65.0	35.0
T2	*E. coli* B	2.3	88.5	65.0	93.0	86.0
T3	*E. coli* B	2.5	61.0	0	70.0	0
T4	*E. coli* B	2.0	98.9	0	99.0	0
T5	*E. coli* B	0.7	74.0	74.0	74.0	71.5
T6	*E. coli* B	3.8	84.7	56.0	89.0	57.0
T7	*E. coli* B	5.1	71.0	0	60.0	0
P22C	*S. typhimurium* LT-2	3.0	91.3	21.7		
C21	*S. typhimurium* SL1069	1.0	92.6	0		

[a] Host cells were mixed with each phage suspension. After incubation at 37 C, the number of free phage was measured by the plaque counting method. Adsorption efficiency was calculated by the method described in the text.

added to the cells previously treated with PLB, three appearances of phage attached to the cells were observed. On the normal sites between the projections, irreversibly adsorbed phage with empty heads and contracted sheaths were seen mainly, with tail core needles clearly penetrating the wall (Fig. 2). Conversely, the majority of phage attached to the projections had full heads and extended sheaths. Few phage attached irreversibly to the projections, and the needles of these phage could not be seen (Table 2).

Effects of polymyxin on cells which had previously adsorbed phage T2 or T4. *E. coli* B cells which were allowed to adsorb phage T2 for 10 min were then treated with PLB for 10 min and fixed. Such cells tended to undergo lysis. This might have been a result of the potentiation of the lytic action of the phage lysozyme by polymyxin (25). In any event, many lysed cells with adjacent phage were seen in every part of the sections, in spite of infection by the same MOI of phage as in the experiment described above. Treatment with polymyxin resulted in the appearance of projections from the wall, except at the sites which had irreversibly adsorbed phage T2 (Fig. 3). Unexpectedly, in the cells which had adsorbed phage T4 before polymyxin treatment, no structural alterations, such as the wall projections, even in the locations between the actual sites of phage adsorption, were produced by polymyxin (Fig. 4).

Effect of polymyxin on adsorption of the phage to purified LPS. LPS was extracted from the cell wall fraction by the hot phenol-water method and purified as described above. The lyophilized LPS was dissolved (100 μg/ml) in modified M-9 medium, and 1 ml of the LPS solution was mixed with 1 ml of each phage suspension containing about 10^8 PFU/ml. After 10 min of incubation at 37 C, the number of free phage was measured by the plaque counting method. As shown in Table 3, purified LPS from *E. coli* B adsorbed phages T3, T4, and T7, but not phage T2. Also, purified LPS from *S. typhimurium* LT2 and SL1069 adsorbed phages P22C and C21. To determine the effect of PLB on the adsorption of phage to LPS, the LPS solution was mixed with a PLB solution at various concentrations for 10 min at 37 C before being adsorbed by phage. Dose response of PLB to the adsorption efficiency of phage T4 to *E. coli* B LPS and phage P22C to *S. typhimurium* LT2 LPS is shown in Fig. 5. A concentration less than 2.5 μg of PLB per ml did not affect 100 μg of LPS per ml, whereas more than 25 μg/ml did. Therefore, 25 μg of PLB per ml was used for pretreatment of LPS. Adsorption of phages T3, T4, and T7 to the LPS of *E. coli* B, phage P22C to the LPS of *S. typhimurium* LT2, and phage C21 to the LPS of *S. typhimurium* SL1069 was prevented by PLB treatment (Table 3).

TABLE 2. *Appearance of phage T2 adsorbed to polymyxin B-treated E. coli B cells*

Appearance of phage	No. of phage attached to normal surface	No. of phage attached to projections
Contracted sheath and empty head	41	5
Contracted sheath and full head	9	3
Extended sheath and full head	1	32
Total	51	40

TABLE 3. *Effect of polymyxin B on adsorption to purified lipopolysaccharide (LPS)*

Phage	No. of original phage (× 10⁸)	LPS from	Adsorption efficiency of phage[a]			
			With LPS		With PLB-treated LPS[b]	
			No. of free phage after adsorption (× 10⁸)	Per cent efficiency	No. of free phage after adsorption (× 10⁸)	Per cent efficiency
T2	1.0	*E. coli* B	1.1	0	1.0	0
T3	3.4	*E. coli* B	0.0073	99.8	3.5	0
T4	1.9	*E. coli* B	0.0087	99.6	2.0	0
T7	6.3	*E. coli* B	0.48	92.3	7.0	0
P22C	2.7	*S. typhimurium* LT2	0.30	88.9	2.5	7.4
C21	7.3	*S. typhimurium* SL1069	0.80	88.8	6.6	9.6

[a] LPS solution (100 μg/ml) was mixed with each phage suspension. After 10 min of incubation at 37 C, the number of free phage was measured by the plaque counting method.
[b] Treatment was with 25 μg of polymyxin B (PLB) per ml.

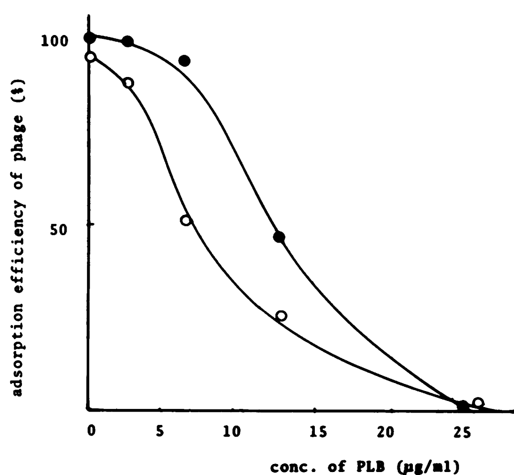


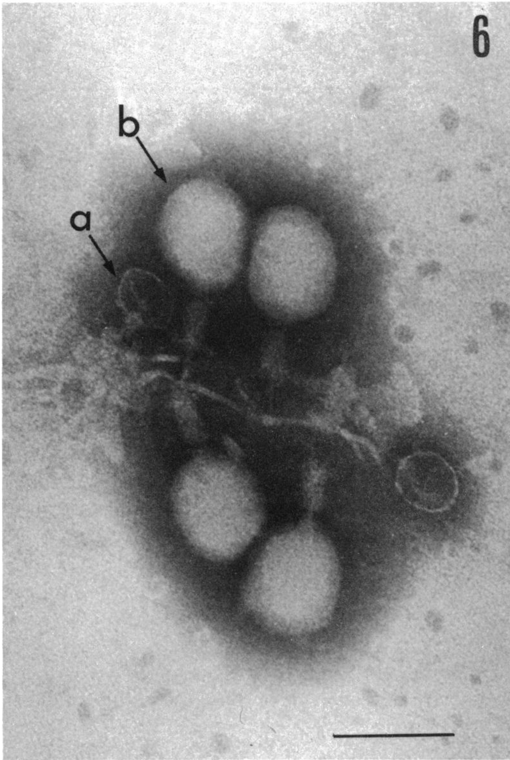
FIG. 5. *Dose response of polymyxin B for the adsorption efficiency of phage T4 to E. coli B LPS (●), and of phage P22C to S. typhimurium LT2 LPS (O).*

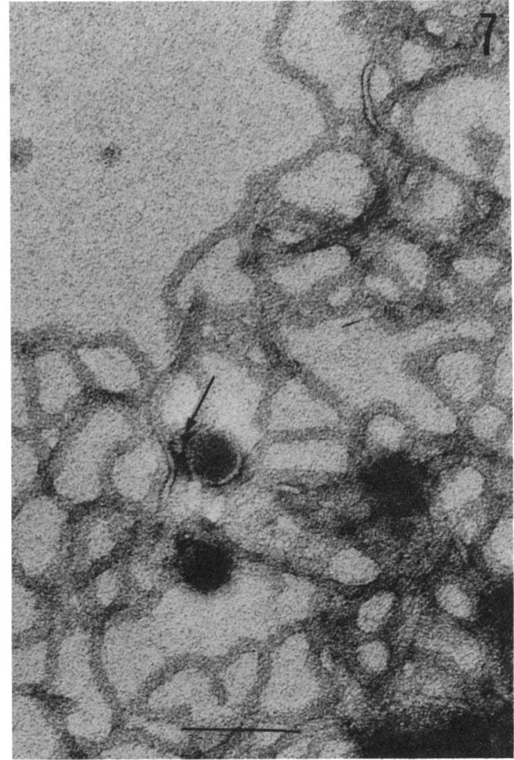
Electron microscopic appearance of phage adsorbed to LPS. The purified LPS polymer, stained negatively, appeared as a ribbon-like structure with frequent branching. Phage T3 adsorbed to LPS through their short tails, and their heads were frequently empty. Phage T4 adsorbed often to both sides of LPS strands at similar distances, and further on the same strand phage T3 adsorbed when the LPS was incubated with a mixed suspension of phages T3 and T4 (Fig. 6). Phage P22C adsorbed by the spikes of their tail plates, but their heads were not empty in the negatively stained preparation. In the preparation positively stained by uranyl acetate, the heads of the phage were strongly stained (Fig. 7). The spikes of the tail plate attached closely to the dense line which was visible in the profile of the LPS strand. Table 4 shows the distribution of the appearance of each class of phage adsorbed to the LPS.

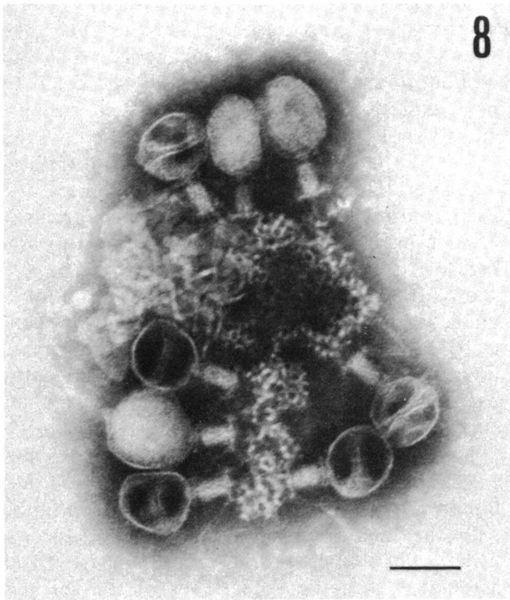
Electron microscopic appearance of LPS treated with PLB. Doses of PLB (less than 2.5 μg of PLB per ml to 100 μg of LPS per ml) which had no effect on phage adsorption did not produce any morphological alterations in the LPS strand. LPS treated with doses of the drug (2.5 to 6.25 μg of PLB per ml to 100 μg of LPS per ml) became coiled loosely or tightly in spiral form and broken down into shorter segments (Fig. 8, 9, 10). In positively stained preparations, the trilamellar structure was clearly visible along the spiral of the coiled LPS (Fig. 11). The loosely coiled LPS was still capable of irreversibly adsorbing phage. After treatment with larger doses of the drug (12.5 to 25 μg of PLB per ml to 100 μg of LPS per ml) the LPS became flat and amorphous. Some flat LPS fused together and became an amorphous mass resembling a membrane. At the surface and inside of the amorphous mass, coiled LPS often appeared (Fig. 12). Otherwise, the flat LPS was broken down into small fragments with irregular shape (Fig. 13), which may correspond to the ones described by Lopes and Inniss (13). The flat LPS may have resulted from the collapse of a trilamellar structure, as indicated by the arrow in Fig. 13. With increasing doses of the drug, only small fragments of collapsed LPS were observable. This collapsed LPS did not adsorb phage.

DISCUSSION

PLB and CL act by damaging the cell envelope (20). We have shown that treatment of gram-negative bacteria with polymyxin results in the formation of projections from the outer layer of the cell envelope and the liberation of cytoplasm through cracks in the membrane (11). Rifkind (17, 18) reported that the lethal activity







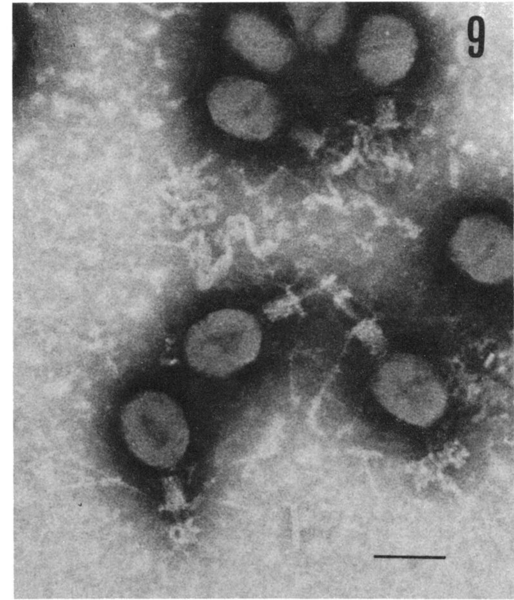


FIG. 6. *Negatively stained phages T3 (a) and T4 (b) adsorbing to a single strand of E. coli B LPS. Markers represent 100 nm.*

FIG. 7. *Positively stained phage P22C adsorbing to S. typhimurium LT2 LPS. The spikes of the tail plate are closely attached to the dense line of the LPS strand (arrow).*

FIG. 8. *Negatively stained, loosely coiled LPS of E. coli B pretreated with 6.25 μg of polymyxin B per ml, allowed to be adsorbed by phage T4.*

FIG. 9. *Negatively stained segmentation of the loosely coiled LPS of E. coli B pretreated with 6.25 μg of polymyxin B per ml, allowed to adsorb phage T4.*

TABLE 4. *Appearance of phage adsorbed to purified lipopolysaccharide (LPS)*

Appearance	T4						T3				P22C			
	In original suspension		+ *E. coli* B LPS		+ PLB-treated[a] *E. coli* B LPS		In original suspension		+ *E. coli* B LPS		In original suspension		+ *S. typhimurium* LT2 LPS	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Full head							149	91.4	30	14.0	138	76.7	170	84.6
Extended sheath	284	98.6	38	12.3	151	100								
Contracted sheath	2	0.7	254	82.4	0	0								
Empty head							14	8.6	184	86.0	42	23.3	32	15.4
Contracted sheath	2	0.7	16	5.3	0	0								
Total	288	100	308	100	151	100	163	100	214	100	180	100	202	100

[a] Treatment was with 25 μg of polymyxin B (PLB) per ml.

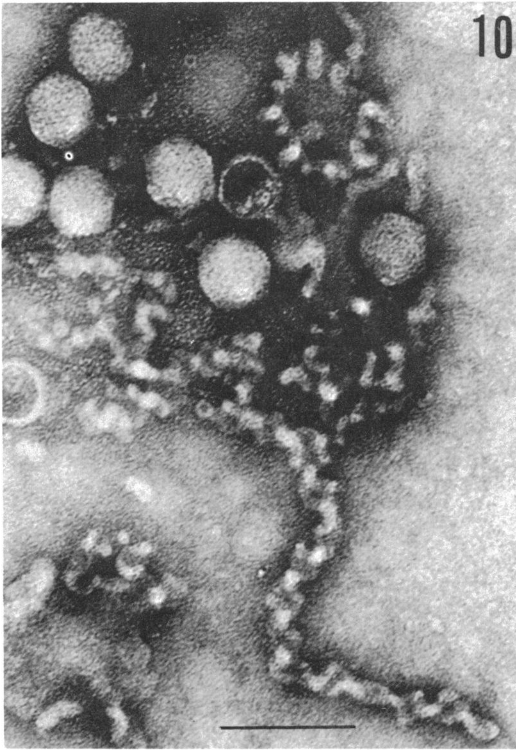
of bacterial endotoxin was much reduced by polymyxin treatment. Recently, Lopes and Inniss (13) showed by electron microscopy that isolated LPS from *E. coli* 018 was broken down into small fragments by polymyxin. The results of our experiments suggest that polymyxin affects the LPS specifically among the cell wall components. The outer surface of the cell wall of *E. coli* B has many kinds of phage receptors. Especially, LPS and lipoprotein of the wall were capable of adsorbing different classes of T-series phage (26). Phages T3, T4, and T7 fix to LPS, whereas phages T2 and T6 fix to lipoprotein. Phage T5 fixes to the lipoprotein containing LPS (27).

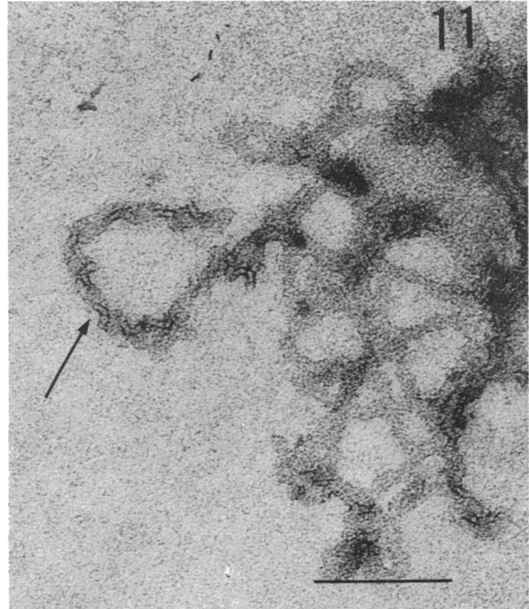
The present experiment indicates that receptors functional for phages T3, T4, and T7 in *E. coli* B and for phage C21 in uridine diphosphate galactose 4-epimeraseless mutant of *S. typhimurium* are specifically lost after polymyxin treatment. Adsorption of phage P22C, which needs repeating units of sugar in LPS as the receptor, was incompletely inhibited by polymyxin treatment.

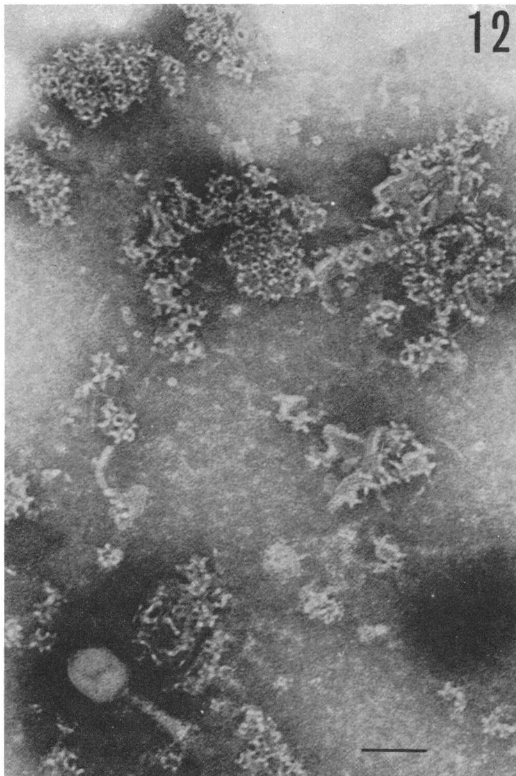
These experimental data indicate that polymyxin exerts its effect by disorganization of the cell wall LPS, although it has not been made clear whether polymyxin affects the polysaccharide part or lipid part of LPS. Observed electron microscopically, phage T2 was found to be irreversibly adsorbed to the smooth wall surface surrounded by projections which were produced by PLB. Almost all phage which attached through their tail tips to the top of the projections had extended sheaths and full heads. A few phage, attaching to the top of the projections, seemed to be irreversibly attached. This appearance may be interpreted to mean that (i) the phage and projections, while actually occupying adjacent sites, were superimposed, or (ii) T2 phage receptors were contained in a few projections. For resolving such a problem, it might be useful to observe the specimen tilted in more than one direction by using a goniometer stage. In the cell adsorbing phage T2 before PLB treatment, projections were produced between the sites where phage had adsorbed irreversibly. This shows that the receptors of phage T2, consisting of lipoprotein, are not affected by PLB. In the next experiment, in which cells which had previously adsorbed phage T4 were used, it was expected that there would be some morphological alterations at the phage-attached sites after PLB treatment. On the contrary, no alterations of such projections could be observed on the surface of the cell wall. Simon and Anderson (22) showed that at the final step of adsorption of T4 phage to the cell wall their tail-core needles penetrated about 13 nm into the cell wall. Accordingly, it might be considered that the receptors had suffered some damage by the phage and could no longer react with polymyxin.

Rothfield et al. (19) reported that LPS may exist in the outer membrane of a bacterium as a bimolecular leaflet interspersed or continuous with the membrane leaflets. However, there is little doubt that LPS and lipoprotein are closely associated with the outer layer of the cell wall (15) and that both are arranged in a single interconnected mosaic pattern, of which the only visible structure in thin sections is a typical unit membrane (4, 7, 8, 10, 23).

From our electron microscopy observations it may be concluded that polymyxin acts to disorganize the LPS in the outer layer of the cell wall of gram-negative bacteria, which is composed of LPS and lipoprotein in a mosaic pattern. The







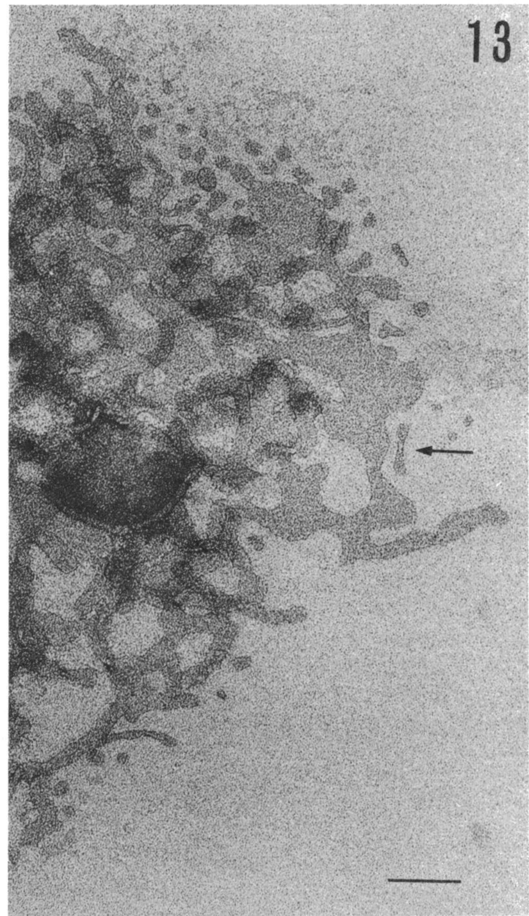


FIG. 10. *Negatively stained, loosely coiled LPS of S. typhimurium LT2 pretreated with 6.25 μg of polymyxin B per ml, to which phage were allowed to adsorb. Markers represent 100 nm.*

FIG. 11. *Positively stained coiled LPS of S. typhimurium LT2 treated with 6.25 μg of polymyxin B per ml (arrow).*

FIG. 12. *Negatively stained fused LPS of E. coli B treated with 12.5 μg of polymyxin B per ml.*

FIG. 13. *Positively stained, flat amorphous mass of collapsed LPS of S. typhimurium LT2 treated with 25 μg of polymyxin B per ml. Collapse of trilamellar structure of LPS strand is indicated by arrow.*

disorganization may result in formation of the projections. On the other hand, the lipoprotein retains its normal functions and structure after polymyxin treatment.

The purpose of the second experiment was to determine whether or not the function of the purified LPS extracted from the cell wall as the receptor of the phage was affected by polymyxin and to observe the morphological alterations of LSP at that time. The purified LPS has a ribbon-like structure, and its profile has a trilamellar structure with two dense outer lines enclosing a less dense core as shown by Shands, Graham, and Nath (21). Phages T3 and T4 specifically adsorb to their respective receptor on the LPS strand. Shands et al. (21) suggested that purified LPS exists as bimolecular leaflets since the ribbon had a trilamellar appearance with two dense outer lines, to which "core" polysaccharide perhaps contributes, and an inner less dense lipid moiety in preparations positively stained by uranyl acetate, when viewed edge on. De Petris (4) reported the same observations in thin sections with regard to the purified LPS extracted from *E. coli* B. Our observation that phage T4 adsorbed to both sides of the LPS ribbon in negatively stained preparations (Fig. 6) and that phage P22C adsorbed to the outer layer of the profile of the LPS in positively stained preparations when viewed edge on (Fig. 7) supports these authors' proposal that the extracted LPS might be a bimolecular leaflet-like structure and also indicates that the outer surface of these bimolecular leaflets might be occupied by phage receptor sites. Treated with low doses of polymyxin, the LPS turns into a spiral form, the profile of which shows the trilamellar structure. These spiral forms of LPS still retain their function as phage receptors. The collapsed LPS, resulting from treatment with high doses of polymyxin, loses the typical trilamellar structure and its function as phage receptor. From these results, it may be concluded that polymyxin acts to disorder the arrangement of molecules in bimolecular leaflets of LPS. However, the solution to the problem of the exact action of the antibiotic will require much additional study. And further, the relationship between the formation of projections from the outer layer of the cell wall and the collapse of the purified LPS by treatment with polymyxin remains a question.

ACKNOWLEDMENT

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